DNA damage and the circadian clock

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Abstract

The role of the circadian clock has already been demonstrated for virtually all physiological processes, but it was only recently shown that cells were more sensitive to DNA damage at specific times of the day; that the peak of synthesis of mRNA and proteins of genes coding for products involved directly or indirectly in DNA repair was differentially timed in different tissues; and that the growth of some types of cancer followed a circadian pattern. The paper reviews the specificities of the clockwork mechanism in living cells associated with repair of DNA damage with regards to its role in ageing and carcinogenesis. The role of heritable polymorhisms and somatic mutations in the risk for development of common diseases (cancer, but also other types of diseases and conditions, such as obesity and metabolic syndrome), their course and the possible outcomes is reviewed in animal models and in man. The circadian oscillations in the levels of major proteins of DNA-damage related signalling and DNA repair are discussed in relation to differential mechanisms of defence against genotoxic damage. The paper outlines the modern concept of 'chronotherapy' - that is, anticancer therapy administered at specific times of the day/ night cycle that could be associated with better outcomes in some patients and analyses the individual variance in the tolerability of chronotherapy vs. maximum-dose anticancer therapy.

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1. Natural 24-hour biological clock

The clock on the wall opposite him had only one hand and no numbers at all.
Written around the edge were things like "Time to make tea",
"Time to feed the chickens", and "You're late!"
J. K. Rowling, Harry Potter and the Chamber of Secrets (1998)

Normal physiology usually has a rhythmical pattern to it, that is, different activities in the living cells normally occur every day and within the limits of fixed time intervals. For example, the morning cortisol release normally occurs between 6 and 8am; the output of diuresis is at its lowest at night and at its highest in the afternoon, the melatonin secretion usually starts at the beginning of the darkness phase (6–9pm), etc.). Circadian rhythm' (from the Latin phrase circa diem', that is, around a day') or circadian biological clock' are terms used to describe the periodically occurring changes in the expression profile, the biochemistry and the physiology of a cell, a tissue or an organism (e.g. the alternating rest-activity cycles; feeding and metabolism rhythms; daily changes in body temperature, pulse rate and blood pressure; nervous system activity; hormone release; elimination of waste, etc.). The levels of the majority of the mRNAs, proteins and metabolites in eukaryotic cells rhythmically rise and fall in a cyclical fashion. The length of the complete cycle is usually close to 24 hours.

According to the definition given by Beatrice Sweeney, one of the pioneers in the field of research of circadian rhythms, *"A circadian rhythm is an oscillation in a biochemical, physiological, or behavioural function which under conditions in nature has a period of exactly 24 hours, in phase with the environmental light and darkness, but which continues to oscillate with a period of approximately but usually not exactly 24 hours"* [1]. Indeed, the biological clock of a cell, a tissue or an organism is normally aligned (entrained) to the rhythmic changes in the environment (daylight/night cycle, changes in the ambient temperature at day and at night, food availability during different phases of the 24-h period, etc.). Normal functioning of the circadian machinery ensures that the cell, the tissue and the organism may continue with their normal activities even in the absence of external cues (see below) referring to what point in the subjective time of the day it is at the particular moment and/or how much time had elapsed between certain time points marked by specific physiological activities. For example, even when housed in complete darkness, normal mice would go to sleep and wake up at approximately the same time within the 24-h cycle. Similarly, the normal locomotor activity of mice housed in the dark would occur within a time period corresponding to the subjective night (as rodents are predominantly nocturnal) and the usual times at which food is normally available would be anticipated (usually manifested by increased locomotor activity preceding the usual time of presentation of food [2, 3].

The phrase biological clock' (often used interchangeably with circadian clock' or circadian rhythm') could be heard so often nowadays, and in contexts so unrelated to science that it is hard to remember that it had always had a strictly defined meaning. In simpler words, it is the biological clock that reminds us that several hours have passed since we have last eaten (feeding time); that 7–9 hours have passed since we have fallen asleep at night (time to wake up); that we have gone without sleep for 16 or more hours (time to go to bed), etc. For some reason, the term biological clock' is popularly (and incorrectly) used as a

connotation of the mechanism of ageing (and most commonly – as a reference to the upper limit of reproductive age of women (e.g. "her biological clock is ticking fast, she is desperate")). In order to avoid any such confusion, we will briefly outline the difference between the basic types of biological clocks.

In principle, a biological clock may belong to one of two basic types: 1) oscillators (as is the circadian clock and similar clocking mechanisms that normally work in cycles shorter than 24-h – 12-h, 8-h, etc. (intradian clocks)); and 2) unidirectional (hourglass) clocks (telomere attrition rate, the Hayflick's limit, etc.). Oscillating clocks usually regulate the normal day-to-day functioning of the cell, the tissue and the organism in young as well as in old age. Indeed, as we will see below, as age advances, the regularity of circadian rhythms becomes impaired and more prone to resetting or disruption by minor stimuli, with multiple deleterious consequences. Unidirectional clocks measure the time until individual cells (and, in a more complex manner – the tissues and the organism) are decommissioned by the mechanism of replicative senescence/death of old age.

Many of the normal physiological processes follow a rhythmical circadian pattern, and many aspects of daily life may revolve around circadian changes. For example, the levels of cortisol in mammals and man peak every day almost immediately after waking up from nighttime sleep, enhancing the stress response in the early wakefulness phase. This may be manifested by heightened arousal, including hypersensitivity to various stimuli and/or hypervigilance (the well-known early morning irritability'). The cortisol awakening response may be enhanced by acute and chronic sleep deprivation (having not slept the previous night or and/or chronic loss of sleep); need to awake very early in the morning (e.g. in shift workers taking very early-morning shifts); consumption of alcohol, caffeine, and other substances; and/or stress, producing anxiety and jitteriness, and, sometimes, volatile temper [4, 5]. Most people experience the afternoon plunge' in concentration, attention span and capacity for goal-oriented activities in the hours roughly between 1 and 4pm. The latter was probably responsible for the establishment of the tradition of the afternoon siesta. The need to rest in the afternoon is often dismissed as effects of lunch, but its effects occur in people that habitually miss lunch as well, therefore, it is not always related to digestion. The body temperature and the arterial pressure usually decrease at night, which may be responsible for subjective feeling of cold' that may develop in people that have stayed up well past their usual bedtime, even in a warm environment. Apparently, the circadian clock cannot be simply ignored or fooled', although, as we would see below, it could be reset (adjusted, entrained). Entrainment of the circadian clock to a different schedule, however, may take considerable time and may sometimes have harmful consequences for the organism.

In higher eukaryotes, including mammals and man, the central circadian time' is set by the master circadian clock. It is located in the suprachiasmatic nuclei (SCN) in the hypothalamus [6, 7]. There are peripheral clocks as well – in the gastrointestinal tract, the heart, the lungs, the kidneys, the peripheral nervous system, and in other organs and tissues. Peripheral clocks manage tissue and organ-specific circadian events. The master clock is usually adjustable by light, whereas the peripheral clocks are dependent on cues other than light [reviewed in 8]. Disruptions of the daily sleep/wake routine may impair the functioning of

the organism more significantly via deregulation of the peripheral clocks than the master clock. This is because the master clock is rapidly adjustable, whereas the peripheral clocks are synchronised with the master clock indirectly, e.g. by hormone-associated and/or neural signalisation. This means that the resetting of peripheral clocks may be significantly offset from the master clock (sometimes by several hours). For example, the peripheral clocks in the gut are responsible for the periodic activity of its different segments, ensuring the timed passage of the contents of the gastrointestinal tract along its length. Thus, the stomach and the small bowel are emptied regularly to allow room and capacity for digestion of new food and the urges for solid waste elimination typically occur roughly at the same time every day [9]. This may explain the common and fairly embarrassing phenomenon of failure to complete habitual acts of elimination of faecal waste after the normal circadian rhythm has been disrupted (e.g. by awakening and arising from nighttime sleep unusually early or unusually late). Similarly, as peripheral clocks in the cardiovascular system normally cause lowering of blood pressure and pulse rate during nighttime sleep and their subsequent increase after morning awakening, disruption of the circadian rhythm may account for the common occurrence of feeling generally unwell/weak' and/or vasovagal syncope after taking a long nap during daytime (pulse rate and blood pressure being too low at times when they are normally higher). A very large time lag exists between the master clock and the peripheral clock in skin (up to 4–8 hours) [10, 11].

Every eukaryotic cell possesses the requisite molecular machinery of the core circadian clock and may follow the physiologic rhythm for a long time, even in the absence of major cues. The maintenance of the circadian rhythm, however, is better sustained on tissue, organ and organism levels than in single cells, as the correspondence between the central and the peripheral clocks is necessary to ensure the subtle adjustments of the circadian rhythm. Typically, the clock of the organism is rapidly reset in response to specific cues, but cultured cells may rapidly lose the circadian rhythm, sometimes even in the presence of potent clock-adjusting cues [12].

The external cues capable of adjusting the circadian clock (also called Zeitgebers', or time givers') may be endogenous or exogenous. A prime example of exogenous clock-adjusting factor is light. The master clock in the mammalian brain is exquisitely sensitive to light. Even a single short light pulse may suffice for resetting the master clock, provided that the wavelength of the light is shorter than the wavelength of infrared light. The light is perceived by the photoreceptors in the retina; then the signal is transmitted to SCN and subsequently to the pineal gland. The latter is the topological site where melatonin is produced. Melatonin (N-acetyl-5-methoxytryptamine – a tryptophan derivative) is the major hormone regulating the sleep-wakefulness cycle as well as other physiological processes, such as sexual development, sexual behaviour, etc. Melatonin is usually produced in small quantities during the daylight phase of the 24-h cycle in diurnal animals and man and in larger quantities during the darkness phase as light suppresses its synthesis [13]. Thus, light at night is likely to keep one awake, although they usually are asleep at this time. Similarly, exposure to an environment that is relatively dark and monotonous (e.g. during travelling, darkened theatre halls, listening to a long and/or dull presentation, etc.) may make one doze off, although it is daytime when the individual is normally awake and

alert. In rodents (which are usually nocturnal) the peak of melatonin synthesis occurs at the end of the darkness phase (onset of rest phase) [14].

Exogenous melatonin may cause sleep phase advancement [15, 16]. Melatonin and/or bright light during the day are sometimes used for targeted entrainment of the sleep-wake cycle towards hours other than the habitual bedtime and morning awakening hours (e.g. in people for which late sleep phase onset and the corresponding late morning awakening clashes with work, family, and other routines). Disturbed melatonin production is a major contributing factor in the pathogenesis and the presentation of seasonal affective disorder and major depressive disorder [reviewed in 17] (for more information see below). Bright light therapy in waking hours may sometimes be used to improve and/or stabilise mood and help sleep at night in the course of treatment of mood disorders [18, 19].

Some hormones may function as endogenous cues for adjustment of the circadian clock. For example, administration of the synthetic cortisol analogue dexamethasone may reset the peripheral circadian clocks in mice [20]. Dexamethasone was shown to affect the expression of the core clock genes in cultured human cells [21]. In female rats, estrogen regulates the expression of *Per1* and *Per2* [22]. Notably, the cited study showed that the effect of estrogen was different in reproductive and non-reproductive tissues of female rats and between the master clock and the peripheral clocks.

The exact time at which a phase (e.g. rest phase/activity phase) occurs in the 24-h cycle may vary (sometimes – significantly) between individuals of the same species. Among humans, there have always been people that feel the need to go to bed early in the evening and wake up refreshed and alert early in the morning (the morning lark' chronotype); whereas others feel their best in the late afternoon and evening, do not go to bed until late after midnight and sleep soundly until noon the next day (the night owl' chronotype). Extreme variants of the morning lark' and night owl' chronotypes may be synonymous to advanced and delayed sleep phase disorder. Misalignment between the time in the subjective day when one really must get up (e.g. to go to school or to work) and the time one feels rested; and between the time when one needs to go to bed because they are too tired to stay awake and the time when they ought to go to bed in order to ensure that they have rested for enough hours may cause problems. The latter, however, usually become significant only in the cases when the time points between "desired time" and "actual time" for a phase switch are desynchronised by three or more hours (e.g. one must get up at 7am to get to work on time, therefore, they ought to be in bed by 11pm; but one can't normally go to sleep before 2am and does not feel their best before noon). Nevertheless, the fact that the timing of the rest/activity cycle may be quite different in different people has been brought to public awareness to the point that more flexibility in planning study and work cycles are currently being advocated to ensure than individuals with either of the basic chronotypes have equal chances for performing well in school, at the workplace, and in specific cases, such as examinations and business meetings [23, 24, 25].

The chronotype is heritable, and in specific cases (see below) the molecular variant implicated in chronotype establishment may be identified. It is, however, moderately changeable and may be learned or 'transitioned to', given enough time. Daily routine may play a major role in the adjustment and even the complete reversal of the chronotype. The

fact remains, however, that the chronotype of parents is often similar or identical to the chronotype of the offspring. The latter formed the basis of a hypothesis about the possibility of assortative mating among people with morning' and evening' chronotypes', based on the assumption that persons with the one chronotype could only seldom meet persons with the other chronotype, as their daily routines would dovetail' rather than match [26].

Frequent changes of the daily routine causing disruption of the circadian rhythm (circadian misalignment) – e.g. travelling across several time zones (jet lag), taking rotating shifts, etc.; acute or chronic stress; or failure to entrain the biological clock because of misguiding environmental cues (e.g. working during the day in badly lit offices and sleeping in the light-polluted environment of large cities) may affect the human organism in many ways. Circadian rhythm disruption may bring about various conditions associated with day/night cycle reversal (chronic insomnia at night and/or daytime sleepiness); disordered food intake and energy expenditure (e.g. undesired weight gain); troubled digestion; hormonal dysregulation and others (for details, see below). Notably, these adverse effects may be independent of sleep deprivation, that is, they may develop even in individuals that get their 7–9 hours of sleep per 24h and their minimum dose of light when they are awake, but these occur 'at the wrong time' of the day. There is also the well-documented condition of 'midwinter insomnia' affecting people living in territories close to the North polar circle, where the sun does not rise above the horizon for weeks and months in the winter [27]. This is believed to be directly related to disordered production of melatonin.

In some cases, disruption of the circadian rhythms may be dangerous. It infamously known that working at night, especially performing monotonous tasks in a badly lit environment (e.g. driving) may result in occurrence of very short (lasting only a second) episodes of falling asleep, which may have very grave consequences. This may occur regardless of whether the driver had slept enough before they sat behind the wheel or not (albeit is it more likely to occur if they have not).

Chronic disruption of the rhythm set by the internal clock may put the individual at increased risk for some common diseases with multifactorial genesis. Circadian misalignment may be associated with increased risk for development of insulin resistance (metabolic syndrome, diabetes type 2) [28]; various cancers (breast cancer, prostate cancer, colorectal cancer, and others) [29–31]; arterial hypertension; cardiac arrhythmia [32–34] – in other words, many of the diseases of middle and advanced age'. When the cancer had already developed, chronic disruption of the circadian rhythms may sometimes accelerate its progression and/or increase the risk for metastasising [30, 35] (for details, see below). Timing of anticancer therapy around the circadian cycle may increase its efficacy and minimise the associated adverse effects [36] (for more information also see below).

2. Looping the loop - core machinery of the circadian clock

On a single-cell level, the functioning of the circadian clock is based on periodical oscillations in the levels of several proteins, forming a negatively controlled feedback loop [37, 38]. Some of the proteins of the core clock machinery function as transcriptional

transactivators (more rarely – suppressors) of the expression of their target genes (clock-controlled genes, CCG). Others are responsible for the correct functioning (closing) of the feedback loop. The mammalian proteins Clock (Circadian Locomotor Output Cycles Kaput), Bmal1 (Brain and Muscle Arnt-Like Protein 1, Arntl1) and Npas2 (Neuronal PAS domain-containing protein 2) represent the first type of core clock proteins (positive regulators). Clock and Bmal1 (or Npas2) function together as a heterodimer, binding to specific sites in the regulatory regions of their target genes and activating the expression of light-inducible (day-phase) genes [39, 40].

The Cry proteins (from cryptochrome') – Cry1 and Cry2 and the Per proteins (from period') – Per1, Per2 and Per3 are negative regulators of the core circadian clock in mammals. Per and Cry proteins also function together, forming heterodimers. The Per/Cry complex switches the expression of Clock and Bmal1 off, downregulating its own transcription as well (Fig. 1) [41, 42]. Per2, Cry1 and Cry2 proteins interact directly with Bmal1, although they bind to different domains in its molecule [41].

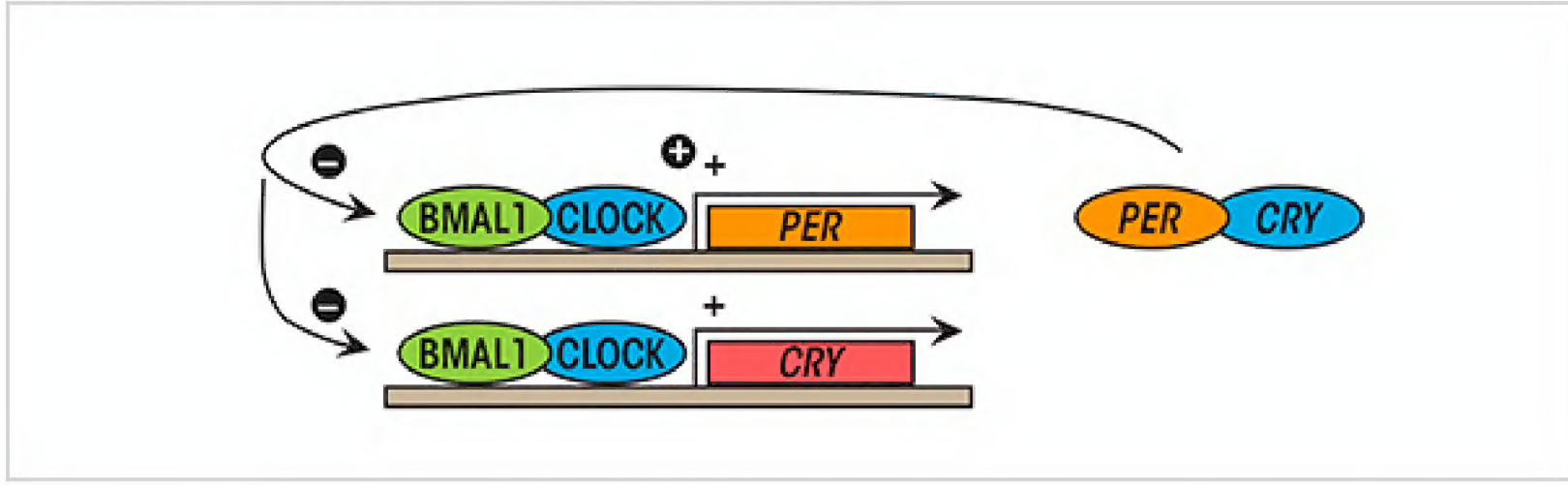

Figure 10. Core feedback loop of the circadian clock in mammals [http://pubs.niaa a.nih.gov/publications/arcr351/87-96.htm].

Bmal1, Cry and Per proteins have half-lives of only about 3 hours, as they are continually tagged for degradation by E3 ubiquitin ligases [43, 44].

Npas2 may substitute for Clock in the suprachiasmatic nuclei [45, 46], whereas peripheral circadian clocks normally utilise Clock for the activation of light-inducible genes.

The promoters of the genes regulated by the circadian clock are usually GC-rich [47]. The regulatory elements that are direct targets for binding of the proteins of the circadian clock may be grouped into two major types – E-boxes (short CG-rich DNA motifs of the type CAYGTG, where Y is C or T, also E'-boxes (CACGTT)) and D-boxes (enhancer elements with the consensus sequence RT(G/T)AYGTAAY, where R is A or G) [48]. The promoters of Cry1, Cry2, Per1, Per2, and Per3 all have E-box sequences [reviewed in 49]. The genes coding for proteins of the core clock and clock-regulated genes may have more than one E-box (for example, the *Per2* locus in the mouse has five E-box sequences, only one of which is in the promoter of the gene) [50, 51]. Clock-regulated genes contain multiple sites for binding of various cis-acting regulatory factors – transcription factors; proteins implicated in chromatin remodelling (e.g. HMG); proteins associated with proliferative signalling (c-Myc, cyclin D, STAT1 and others) [47]. Many of these cis-acting proteins are, in turn, regulated by the core clock machinery (see below).

Clock and Bmal1 proteins are members of the bHLH-PAS family of proteins. They contain a basic helix-loop-helix (bHLH) domain at the N- terminus [reviewed in detail in 52] followed by two PAS (PER-ARNT-SIM) domains and a PAC (PAS-associated C-terminal) domain [53–56]. The PAC domain contributes to the PAS domain fold, forming a PAS/PAC domain, commonly seen in sensor and signal transduction proteins [reviewed in 57]. Eukaryotic PAS/PAC domains were proposed to have originated from the yellow photoactive protein that serves as a blue-light sensor in bacteria [58, 59]. Npas2 is also a bHLH-PAS protein [60]. Period (Per) proteins also possess conserved PAS domains [61]. Cry proteins have no PAS domains.

The levels of Clock normally oscillate within the 24-h cycle in eukaryotic cells, but the mechanisms for the rhythmical increase and decrease of the levels of Clock mRNA and protein may be different in different species. The levels of expression of Clock remain relatively steady throughout the 24-hour period in the mouse, but the levels of its mRNA and protein may vary significantly between the nuclear and the cytosolic compartment at different times of the day [62–64]. In the SCN of mice (which are nocturnal) the levels of Clock protein in the nucleus are lowest during the day (morning until early afternoon), in anti-phase with the cytoplasmic levels of Clock (which are in this period at their highest levels) and vice versa – in the levels of Clock in the nucleus peak at night, which coincides with their trough level in the cytoplasm [63]. The expression of Bmal1 in the rat suprachiasmatic nucleus peak late in the subjective night whereas in other (light-insensitive) parts of the brain the peak of expression of Bmal1 occurs during the subjective day [65]. In sheep, the level of expression of Clock oscillates in phase with Bmal1 and in antiphase with Per and Cry proteins [66].

The Per/Cry dimer usually suppresses the transcription of day-phase' proteins by binding to their E-box sequences. The peaks of expression of Per and Cry usually occur in anti-phase to the expression peaks of Clock and Bmal1 (Npas2), although the timing may be different for different proteins. In rats *Per1* mRNA peaks in early morning, *Per3* mRNA – late in the afternoon and *Per2* mRNA – in the transition period between the day and the night (at dusk) [67, 68]. In studies of human brains, *PER1* expression was demonstrated to peak soon after sunrise, *PER3* – around midday, and *PER2* in the late afternoon, that is, close to the timing of expression of rodent Per proteins, despite the differences in the timing of activity/rest phases in the two species [69]. Per mRNA peaks in the peripheral clock systems approximately 4h later than the subjective time of its peak in the SCN. Rodent Per2 protein contains a nuclear localisation signal [70]. The regulatory functions of the Per/Cry complex in the SCN are directly dependent on the nuclear entry activity conferred by Per2 [70, 71].

Cry1 and Cry2 may have light-dependent as well as light-independent regulatory functions. The *Cry1* gene contains a day-time' element (D-box) as well as night-time' elements (Rev-ErbA/ROR response elements, RRE) [72]. Mouse Cry1 peaks during the subjective day phase (the rest phase for rodents) and is lowest at night (the activity phase) [73], whereas human Cry1 is predominantly expressed in late evening and night [72].

The proteins of the eukaryotic circadian oscillator are highly conserved, even between distantly related species. Eukaryotic cryptochromes are related structurally and phylogenetically to Class I prokaryotic photolyases [74, 75]. Unlike photolyase where the

FAD is in the core the protein molecule, in Cry proteins the flavin cofactor is contained in a shallow pocket, accessible from the surface of the molecule [74]. Human CRY1 protein has 73% amino acid identity with human CRY2, 48% identity to (6-4)-photolyase of Drosophila and 25% to blue-light protein receptors of plants [74, 76, 77]. The PAS domains of human PER3 share about 50% homology with the PAS domains of human PER1 and PER2 and 30% identity with the PAS domains of the period protein of Drosophila [78].

Besides the core clock machinery, there are also other proteins functioning in the regulation of the circadian rhythm in higher eukaryotes – e.g. Tim1 (Timeless); deleted in oesophageal cancer (Dec) – Dec1 and Dec2; nuclear receptor subfamily 1 group D (Rev-Erb) proteins; retinoic acid-related orphan receptors (RORs), and others [79–82]. Timeless is an accessory factor that suppresses the transactivation of Per1 by Clock/Bmal1 or Clock/Npas2 dimers [79]. The transcription of Tim1 is stimulated by Clock/Bmal1. The levels of Tim1 are normally coupled with the cell cycle phase; with peak levels reached in S phase [83]. Dec1 and Dec2 proteins are bHLH proteins, expressed in the suprachiasmatic nuclei. They too act as transcriptional repressors of Clock/Bmal1-induced transactivation of the Per1 promoter by competing with Bmal1 for the E-box elements in the Per1 promoter as well as by direct interaction with Bmal1 [65, 80]. Rev-Erb proteins (nuclear receptor subfamily group D members) are transcriptional repressors of the expression of the core clock genes Bmal1, Clock and Cry1 [81, 84]. They also regulate the expression of genes coding for products implicated in the metabolism of lipids and bile acid metabolism, adipogenesis and gluconeogenesis [85]. RORs are DNA-binding proteins regulating the transcriptional activity of Bmal1, Npas2, Clock, and Cry1 as well as of other RORs and the Rev-Erb subfamily of nuclear receptors [86–88].

There are usually several hours of delay between the peak in the mRNA level of core clock genes and the peak of the expression of the corresponding protein. The core clock feedback loop is controlled predominantly by the slower transcription-mediated mechanism, as the associated mRNA and protein peaks occur within several hours of each other and the timings of peak and trough levels are normally predictable. However, the core proteins of the clock machinery are subject to modification at post-translational level [63] and some of them are capable of modifying other proteins (for example, Clock possesses acetyltransferase activity [89]). The circadian clock may need to be rapidly reset in response to specific cues (e.g. after a single light pulse or a change in ambient temperature) without having to wait for the transcription and translation of the relevant protein/s. In this cases, the reset may be implemented by the relatively faster post-translational mechanisms (phosphorylation, ubiquitination (and subsequent degradation), SUMO-ylation, etc.) [43, 90]. Clock resetting may also be implemented via transcriptional control. For example, 60 min after the provision of a feeding cue the expression of *Per2* and *Dec1* in the mouse liver was upregulated, inducing a phase shift [91]. Later, the levels of the transcripts of *Bmal1*, *Cry1*, *Per1*, *Per3*, *Dec2*, and *Rev-ErbA* also changed to reflect the phase change. According to this study, *Per2* gene was most sensitive to feeding cues among the core clock genes, as its upregulation could be induced by a minimal amount of food.

Circadian cycles have probably arisen early in evolution, as all eukaryotic cells and even some prokaryotes (e.g. Cyanobacteria) exhibit a 24-h rhythmicity in many of their functions.

Cyanobacteria are among the oldest organisms currently living on Earth. The Cyanobacteria system for maintenance of the circadian cycle consists of only four proteins – the three proteins of the kai cluster (kaiA, kaiB, and kaiC) and the sensory histidine kinase sasA [92, 93]. The prokaryotic and the eukaryotic systems for maintenance of the circadian rhythms are somewhat similar, but not identical. In both types, the regulation of the circadian clock is implemented by feedback loops and the oscillations of the mRNA and the proteins of the core clock machinery are dependent on the phase of the cell cycle and the presence of DNA damage [94, 95]. However, no eukaryotic homologues of the kai genes have been identified yet. Virtually all gene promoters in Cyanobacteria are regulated by the circadian clock, as their survival is directly dependent on the light-dark phase change, but only about 10–15% of all eukaryotic genes are regulated by the circadian clockwork at any given moment [96, 97].

3. Circadian rhythm of the cell cycle

Lente currite noctis equi.

Publius Ovidius Naso, Amores, c. XVI century BC, Book I, XIII, Line 40.

The synthetic phase of the cell cycle in eukaryotic cells occurs predominantly at night (although this may vary in different tissues). Timing of replication to the darkness phase of the day/night cycle is believed to be an adaptive mechanism originating from the early times of eukaryotic life on Earth (the 'escape from light' hypothesis) [98, 99]. The molecule of DNA is particularly vulnerable to damage during replication, as it is free from the protective proteins normally bound to it, the superhelical structure is partially relaxed and there are extensive regions in which the individual strands are separated from each other. Presumably, DNA replication in daytime, when solar radiation was abundant, increased the risk for occurrence of DNA damage in early eukaryotic organisms that were no better equipped than bacteria when it came to protection from electromagnetic radiation. As the organisation of the bodies of eukaryotic organisms became more and more complex, the majority of the cells inside the body became inaccessible to light, but maintained the day-night rhythm, using adapted versions' of proteins that were once used as light sensors and/or proteins of repair of specific types of photodamage (e.g. cryptochromes).

The circadian rhythmicity of entry and exit from the different phases of the cell cycle is especially noticeable in tissues with rapid turnover. For example, dividing cells from colonic mucosa normally enter the G1 phase of the cell cycle in a relatively coordinated fashion in late afternoon/early evening and the percentage of cells in S phase peaks around midnight [100].

There are tissues in which the different phases of the cell cycle occur at times inconsistent with the general rule that replication occurs at night. For example, in tissues that are well protected from genotoxic effects (e.g. haematopoietic progenitors in the bone marrow - protected by layers of connective tissue, fat and bone and very rarely coming in contact with genotoxic agents) the replication is normally timed during the day. This may be observed in species with differently timed activity/rest cycles. For example, the synthetic phase of the cell cycle in the bone marrow of mice occurs predominantly in the morning,

with the mitotic peak occurring in the interval 6am–12am [101]. In human haematopoietic progenitors the peak of replicative DNA synthesis is around noon and in the early afternoon [102]. Notably, the targeting of newly produced blood cells to various peripheral organs and/or back to the bone marrow also follows a circadian rhythm. For example, the reticulocyte levels in peripheral blood of mice were shown to be highest at 8am [101]. The recall of aged neutrophils to the bone marrow and their clearance is specifically timed to the end of the resting period in mice (late afternoon/evening) [103]. The physiological oscillations of the levels of Bmal1 are directly associated with the circadian variations in the trafficking of monocytes to the sites of inflammation in mice [104]. The same authors demonstrated that deletion of *Bmal1* in cells of the myeloid lineage in mice disrupted the rhythm of monocyte trafficking and increased the risk for development of acute and chronic inflammatory disorders.

The skin, as the outer border of the multicellular body, is subjected in full to the environmental changes typical of the day/night cycle (ambient temperature, levels of UV irradiation, etc.). The proliferation of the progenitor cells in adult epidermis (healthy as well as damaged) occurs in an oscillating fashion, with the completion of the cell cycle significantly accelerated (3–5 times) in the early phases of skin regeneration. In the mouse epidermis, the S-phase occurs predominantly late into the night [105], which is consistent with the escape from light' hypothesis. The proportion of S-phase cells in human epidermis is lowest in the morning hours (6–8am), then increases to reach a peak later in the day (in the afternoon – around 3–4pm) [106, 107]. The latter is believed by some authors to contribute to the risk of skin cancer [105, 108].

Melatonin is a potent free radical scavenger [109]. As it is produced at night, when is the peak of the S-phase of many cells types, this function of melatonin may be viewed as an additional defence mechanism against damage to DNA at times when it is especially vulnerable.

The expression of the genes of the core circadian clock may be developmentally regulated. The cell cycle of undifferentiated cells (e.g. cells of the early embryo, pluripotent stem cells) is probably not controlled by the circadian clock, at least until the onset of differentiation. In mouse embryonic stem cells, the rhythmicity of metabolism is established before the rhythmicity in the expression of the core clock genes [110]. The genes of the circadian clock, however, may have other roles in early embryogenesis, as embryoid bodies formed from embryonic mouse stem cells with the *Tim*1 gene knocked out failed to cavitate [111]. The differentiation status of the cells in the adult body may reflect on the expression of the core clock genes. Studies of the expression of the core clock genes in primitive haematopoietic stem cells show that *Per2* is expressed in a rhythmical fashion whereas *Per1*, *Rev-Erb* and *Bmal1* are not [112].

The timing of the phases of the cell cycle of adult stem cells and progenitor cells also follows a circadian pattern. The rhythmic waves of transcription of the core clock genes in adult epidermal stem cells and in dividing keratinocytes coincide with peaks of expression of specific subsets of genes involved in the proliferation and differentiation of keratinocytes (e.g. KLF-9) and in their capacity to respond to environmental cues (e.g. TGF-beta) [113, 114]. Some of the genes coding for products responsible for keratinocyte proliferation and

differentiation are directly controlled by the circadian clock. Such is, for example, the human *KLF9* gene. It possesses several E-boxes to which BMAL1 binds with different affinity depending on the time in the subjective day [113]. In murine hair follicles were identified discrete stem cell populations that were at opposite phases of the circadian clock and responded differently to clock-adjusting cues [115]. Human hair follicles showed oscillating circadian changes in the expression of core clock genes that varied with phase in the hair cycle' [116]. According to the same authors, knockdown of either *BMAL1* or *PER1* in growing human hair follicles was shown to prolong the anagen phase (phase of active growth), making *BMAL1* and *PER1* potential targets for modulating human hair growth.

Undifferentiated cells from rodents and stem cells from primates and man may exhibit very different properties, specifically in the pattern or repair of DNA damage and the cell cycle [117, 118]. As these two are tightly linked with specificities in the maintenance of the undifferentiated state and differentiation, it might be expected that what is valid for the mouse and rat stem cells may turn out not to be valid for human stem cells, or, at least, not completely.

Cancer stem cells may retain the circadian rhythm of cell division, but the properties of the circadian rhythm in them may be somewhat different from the properties in normal cells. Murine cancer stem cells do not exhibit the typical rhythmical pattern of localisation of Per2 to the cell nucleus [119].

Differentiation of precursor cells may also follow a circadian pattern. In mouse oligodendrocyte precursors the cell division occurred predominantly during the rest (daylight) phase, whereas the cell differentiation occurred during the wakefulness (nighttime) phase [120]. In the subgranullar zone of the hippocampus, where adult neurogenesis occurs, the proliferation of neural progenitor cells is controlled by Bmal1 and Per2 by timing of the entry and the exit from the cell cycle [121]. Specifically, *Per2* knockout resulted in suppression of the division of quiescent neuronal progenitor cells, whereas the inactivation of Bmal1 caused delayed exit from cell cycle and high rates of proliferation of neuronal progenitors. These and other findings formed the basis of the theory that sleep deprivation and/or sleep fragmentation may actually inhibit neurogenesis *in vivo* [122].

The expression of core clock genes and clock-controlled genes in cancer cells may significantly deviate from the typical pattern of expression in normal cells. The expression of some of the genes of the core clock machinery may follow a circadian pattern different from the rhythm of nontransformed cells, others may be homogeneously expressed; or their expression may be inhibited or altogether lost (for details see below). Early research in biopsy samples from different types of tumours showed that some types of tumours (e.g. squamous carcinomas in mice and colorectal carcinomas in man) exhibited no variation in the growth rate within the 24-h cycle [123]. In the majority of tumours, however, the mitotic activity has not one, but two peaks per 24 h, the larger one occurring late at night (midnight–2am), and a smaller peak occurring approximately with 12 hours offset from the major peak (around midday) [124, 125]. The timing of the different phases of the cell cycle in cancer cells may be very different from the timing in normal cells of the same tissue. For example, the peak in the DNA synthetic activity in ovarian carcinoma cells is timed around late morning/midday, whereas the peak of DNA synthesis in normal mesothelial cells from

the peritoneal cavity typically occurs in the late evening [126]. In non-Hodgkin lymphoma the peak of DNA synthesis in cancer cells typically occurs at night, whereas the peak of DNA synthesis in normal cells is normally reached in early afternoon [127]. The knowledge about the differences in the timing of the S-phase between normal and cancer cells may be used in modern anticancer therapy to achieve minimum adverse effects and/or better response (cancer chronotherapy – for details, see below).

4. Presence of damage in DNA is a potent cue for adjustment of the circadian clock

Persistence of unrepaired damage in DNA in a normal (nontransformed) cell, preparing for division, results in cell cycle arrest, damage assessment, and attempts for repair of damage and/or induction of apoptosis. Acknowledgement of the presence of unrepaired damage in DNA may trigger adjustment of the circadian clock to a time in the 24-h cycle when the routine checks on DNA integrity, damage repair and/or induction of apoptosis are normally carried out (phase shift). Only after the damage is repaired the cell may attempt to pass the cell cycle checkpoints again.

The first experiments showing the potential of DNA damage as a trigger for phase shifting were carried out on dinoflagellates (single-cell phototrophic eukaryotes capable of producing light) – specifically, Gonyaulax polyedra. Irradiation of G. polyedra cultures with white light or monochromatic light from the visible spectrum during the 'low luminescent capacity' phase (daytime) results in enhanced luminescence in the 'high luminescent capacity phase' (at night), with the rhythm continuing steadily for several days when the cultures were kept constantly in the dark, although the amplitude of the rhythm declined after 3–4 days of constant darkness [128]. The peak luminescence values in the high-luminescence' phase were reached at different times in irradiated cultures and in unirradiated controls, with even a single light pulse capable of resetting the time at which peak luminescence normally occurred [128, 129]. Specifically, the peak of luminescence in unirradiated controls occurred almost uniformly in the middle of the night and the maximum rates of cell division occurred at the end of the night phase. In irradiated cells, phase shifts (advancement as well as delays of the time when the next phase – high luminescent capacity phase – normally occurred) were clearly detectable, varying between 0 and 6 hours in magnitude [129]. Magnitudes of the phase shifts were highest when the wavelengths of light were close to these of blue (475nm) and red-orange (650nm) light, with the latter wavelength being significantly less effective in producing phase shifts than the former [128]. Later, it was observed that irradiation with UV only (wavelength ≈254nm) rapidly produced a decrease in the luminescence levels in the high luminescence capacity phase' and suppressed cell division immediately after irradiation [130]. The capacity for luminescence was rapidly recovered when the cells were illuminated with light from the visible spectrum for several days. In cells that were allowed to recover their luminescence capacity, a phase shift of several hours became apparent, advancing the next circadian phase, with the magnitude of the phase shift roughly proportional to the dosage of UV that the cells had received (the larger the dose, the larger the magnitude of the advancement).

Largest phase advancements occurred when the irradiation with UV and visible spectrum light occurred early in the night phase, and the phase change occurred earlier in cells irradiated with UV light than in cells treated with light from the visible spectrum. Finally, the authors noted that while irradiation with visible light caused phase advances as well as phase delays, depending on the exact time in the circadian cycle when the exposure occurred, irradiation with UV produced only phase advancements. At the time (the early 60-ties), the studies of DNA repair were still in their beginnings, but Beatrice Sweeney and her colleagues proposed that "*...the effectiveness of ultraviolet light suggests that this difference lies in the protein or nucleic acid which thus must form part of the clock machinery*" [130]. Much later, it was discovered that some of the yeast genes, mutations in which caused phase shifts, actually coded for key components of the cell cycle checkpoints (for details, see below). Damage-associated adjustment of the biological clock is seen in all eukaryotic organisms, and DNA damage is currently considered a universal Zeitgeber' for the circadian rhythm [131, 132].

Both major damage-associated signalling pathways (p53-associated and ATM-associated) are capable of induction of circadian phase shifts. DNA damage normally dealt with by activation of ATM/ATR-dependent pathways (strand breaks) usually produces advancement of the next phase of the circadian cycle, with the magnitude of the advance dependent on the subjective time of day when the damage occurred. The mechanism is conserved between different species.

In Neurospora crassa, treatment with DNA damaging agents causes advancement of the next circadian phase [133]. The main proteins responsible for this are prd-4 (an orthologue of mammalian Chk2) and frq, a negative regulator of the core clock feedback loop in yeast. prd-4 is a protein with kinase activity that transmits the signal for presence of DNA damage to the circadian clock. Similarly to Chk2, prd-4 is phosphorylated in the presence of double-strand breaks in DNA [133, 134]. In eukaryotic cells phosphorylated Chk1 and Chk2 (prd-4) phosphorylate the Cdc25c phosphatase, responsible for the removal of the inhibitory phosphate from the CDK1/cyclin B complex [135]. Phosphorylation inactivates Cdc25c and/or enhances its export from the nucleus, thereby blocking the exit from the G2 phase [136–138]. Phosphorylated prd4 also promotes the phosphorylation of frq, resulting in the subsequent degradation of the latter, which effectively shifts the circadian phase to the time of the day when the levels of frq are naturally low (the daytime phase) [133]. If the DNA damage occurred when the levels of frq were high (late in the night), the advancement was usually small (as they were due to decline soon anyway), whereas damage that occurred around the time when frq was being synthesised (early in the night) produced significantly greater phase advancements [133]. frq mutants of Neurospora crassa exhibit circadian rhythmicity with a cycle several hours shorter or longer than wild type N. crassa, depending on the direct effect produced by the mutation (increasing/decreasing the rate of phosphorylation of frq) [139]. prd-4 mutant strain of N. crassa carries a gain-of-function mutation, conferring constitutive activation of the *prd-4* gene (even in the absence of DNA damage) [133]. *prd-4* mutant N. crassa strains fail to respond to clock-resetting cues (light) [139] and exhibit shorter circadian cycle at ambient temperature of 25°C, with the duration

of the cycle shortening progressively as the temperature rises [133]. Conceivably, double mutants (prd-4 mutant/frq^{null}) do not exhibit cycle shortening following DNA damage.

Per1 and Per2 proteins of the mammalian core clock play important roles in the regulation of ATM-Chk2/ATR-Chk1-associated damage response pathways [140, 141]. Repair proteins with signalling and effector functions are activated in the presence of DNA damage. ATM phosphorylates Chk2, whereas ATR phosphorylates Chk1 [142]. Catalytically active Chk2 acts upstream of p53, stabilising it by phosphorylation and eventually producing G1 cell cycle arrest [143]. Chk1-mediated damage-associated pathways typically cause cell cycle arrest in S and G2/M phases of the cell cycle [144]. The accessory clock protein Tim1 suppresses the activation of Per1 by the Bmal/Clock heterodimer and is required for the ATM-dependent Chk2 activation and G2/M phase cell cycle arrest [79, 145]. In mouse cells treated with agents causing double-strand breaks in DNA (readily recognisable by ATM-dependent pathways) Per2 was rapidly degraded following DNA damage, resulting in advancement of the next circadian phase (when the levels of Per proteins were normally low). The magnitude of the advancement was greatest when damage occurred during the synthesis of Per2 (in the late afternoon) and smallest at the peak of Per2 protein levels (late in the night) [132]. The same study demonstrated that UV irradiation (resulting in lesion triggering the ATR-mediated response pathways) also caused phase advancement in mouse cells, with the subjective time of day at which the damage occurred resulting in different magnitude of the advance.

Damage that activates the p53-regulated pathways also produces phase advancements. Some of the core clock genes are directly regulated by p53. For example, the promoter of *Per2* contains a conserved p53-response element that partially overlaps the E-box where the Bmal1/Clock dimer normally binds. In the presence of damage, p53 bound to the response element in *Per2* gene blocks Bmal1/Clock dimer binding to the *Per2* promoter, inhibiting the expression of Per2. This results in advancement of the next circadian phase (in which the levels of Per2 are naturally low) [51]. In cultured p53-deficient cells the amplitude of the normal circadian oscillations is decreased when compared to the amplitude of the oscillations in normal cells [146, 147].

Many of the p53-associated effects on the circadian cycle are exerted indirectly. The transcription from the *Bmal1* promoter was found to be enhanced in the presence of DNA damage, advancing the onset of the next circadian phase [132]. The magnitude of this enhancement, however, was not dependent on the time of the day when the damage occurred, probably because of the fact that Bmal1 is a late responder after occurrence of DNA damage (its expression peaks several hours after the damage has been inflicted) [132, 148].

p53-deficient mice are tumour-prone and short-lived. They cycle normally between the rest/activity phases when housed in complete darkness but the period is shorter than 24h and may be unstable. Light pulses normally rapidly adjusts the core clock in mice kept in complete darkness, causing phase advances or phase delays, depending on the time in the circadian cycle when the light pulse was administered (e.g. a light pulse administered during the subjective night may cause advance of the next (daylight) phase whereas a pulse administered at the end of the subjective day may delay the onset of the nighttime phase).

p53-deficient mice exhibited longer phase delays in response to a light pulse than wild type mice and failed to respond to light with phase advances [51].

5. Circadian oscillations in the levels of gene products directly involved in the regulation of cell division and/or the transition through major cell cycle checkpoints

The stars move still, time runs, the clock will strike.
The devil will come, and Faustus must be damn'd.
Christopher Marlowe, Doctor Faustus (1604), Scene XIV, Line 36.

The levels of expression of many proteins associated with checkpoint transition, progression in the cell cycle and/or induction of apoptosis may vary within the 24-h cycle. Among the clock-regulated proteins are the c-Myc family of DNA-binding proteins; checkpoint kinases, cyclins and other positive regulators of the cell cycle; negative regulators of the progression in the cell cycle – e.g. CDK inhibitors (p21 (Waf1), p16 (INK)) and Gadd45; proteins functioning in DNA damage-associated response pathways and the maintenance of genomic integrity (ATM, p53, HMG-family of proteins); pro-apoptotic proteins (e.g. the pro-apoptotic members of the BCL-2 family – Bax, Puma); and others. The expression of these proteins may be directly regulated by the core circadian clock (usually, via E-boxes – e.g. c-Myc, Xpa, and others), or they may be downstream targets for transactivation by the core clock genes, their levels following (usually, with only a small delay) the oscillation pattern of the levels of the core clock proteins.

The expression of p53 does not follow a circadian rhythm, as p53 is a major player in many and varied processes in living cells and must respond differentially to events causing its stabilisation and accumulation [147, 149]. Nevertheless, its expression may be stimulated or suppressed in response to variations in the levels of the proteins of the core circadian clock. Daytime-phase clock proteins (specifically, Bmal1) generally cause upregulation of the expression of p53, whereas nighttime-phase proteins (Cry1) downregulate it [147, 150, 151]. This is consistent with the escape from light' hypothesis, with p53-dependent G1 checkpoint damage assessment and repair carried out at day and the S-phase at night. For similar reasons, the expression of ATM, the other major damage sensor and effector molecule, is not a subject to direct circadian regulation as well, although Per1 interacts directly with ATM and its downstream target Chk2, activating the ATM-dependent DNA damage response pathways [99].

The expression of c-Myc protein is controlled directly by the proteins of the core clock. c-Myc has affinity to E-box sequences in its target proteins [152]. The promoter of the murine c-Myc gene contains an E-box, where the Clock/Bmal1 or the Clock/Npas2 complexes normally bind, inhibiting the transcription of c-Myc [153]. In Per2-deficient mice, the levels of Bmal1/Npas2 or Bmal1/Clock are reduced, resulting in increased levels of expression of c-Myc and deregulation of the p53-mediated mechanism for induction of apoptosis [148, 154].

The expression of cyclins oscillates within the circadian cycle. The levels of expression of cyclins D1 and E in colonic mucosa typically reach their peak levels in late afternoon/early evening (6pm) before G1/S checkpoint transition; and their lowest levels around midnight, when the M phase is about to begin [100]. The same study reported that the expression of p16 and p21 was at its lowest around midday (for p16) and at 6pm (for p21, respectively). The expression of cyclins may be directly regulated by the clock proteins. Dec1 is known to bind directly to a specific Dec1 response element within the regulatory region of the cyclin D1 gene, inhibiting its expression [80, 155]. The levels of cyclin B1 and its pairing partner Cdk1 (functioning in M phase) and cyclin A2 (pairing partner of Cdk2 and responsible for the progression through S and G2 phases of the cell cycle) are deregulated in mice deficient in Cry proteins [156]. The expression of the negative regulator of the cyclin B/cdc2 complex Wee1 is also directly regulated by the core clock proteins. Wee1 promoter is activated by the Clock/Bmal1 dimer and inactivated by the Per/Cry complex [156, 157].

The expression of some of the essential proteins of the complexes recognising DNA damage and implementing DNA repair may vary rhythmically within the 24-h cycle [147, 158, 159]. Of all XP genes of NER, *Xpa* is the only one that is directly controlled by the circadian clock. Its promoter contains two E-boxes [147, 158]. The levels of *Xpa* mRNA and protein oscillate in a circadian fashion in the mouse brain and liver, with an almost 10-fold difference between the highest and the lowest levels [158]. The peak levels of Xpa are normally in anti-phase with the levels of Cry1 (generally, Xpa levels are high at daytime, when pre-replication checks for genome integrity and DNA repair are carried out; and low at night, when replication occurs) [158]. Xpa is expressed constitutively and at high levels in *Cry1-/Cry2-* double mutant mice [160].

Proteins functioning in DNA repair other than NER may be controlled by the circadian clock. The levels of N-methylpurine DNA glycosylase (a BER glycosylase, excising 3-methyladenine and 7-methylguanine from DNA) and O^6-methylguanine-DNA-methyltransferase (an enzyme catalysing the direct repair of methylated bases by transferring the methyl groups to its own molecule) also oscillate within the 24-h cycle. The peak and the trough levels of these enzymes, however, rarely differ by 1.5–2 -fold [161, 162].

Some of the core clock proteins may directly influence the chromatin architecture and dynamics. Clock protein possesses intrinsic histone acetyltransferase activity, acetylating the conserved Lys14 residue of histone H3 as well as other (nonhistone) targets, including its own pairing partner Bmal1 (again, on specific lysine residue – Lys537) [89, 163]. In mice, Bmal1 acetylation oscillates within the 24-h cycle, mirroring the downregulation of transcription of clock-controlled genes [89, 164]. Acetylated Bmal1 presents a recruiting signal for Cry1 as well, ensuring that the negative feedback control loop is functioning correctly.

The levels of some of the proteins functioning in the maintenance and the remodelling of the chromatin structure, such as HMG, may rise and fall rhythmically in the 24-h cycle. Hmgb1 levels oscillate in the retinal photoreceptor cells in rats, with protein levels peaking at midday and reaching their minimum late at night [165]. Hmgb1 protein normally co-localises in the nucleus together with acetylated histone H3 (a direct acetylation target of Clock) in rat photoreceptor cells [89, 165]. Acetylated Lys14 is a mark of open' chromatin

structure [166]. It is possible that the levels of proteins responsible for the maintenance and the dynamics of chromatin structure, such as HMGB, are regulated in circadian fashion in order to ensure that the chromatin structure is relaxed at times when transactions involving DNA are routinely carried out.

The levels of Gadd45α and Gadd45β (proteins involved in damage-associated inhibition of the entry in S-phase of the cell cycle and induction of apoptosis) also oscillate rhythmically during the day [97, 148]. The expression of GADD45 proteins is believed to be regulated directly by Clock, although only the regulatory sequence of *GADD45B* contains an E-box [97].

The levels of pro-apoptotic proteins and proteins of DNA damage-associated response normally oscillate within the 24-h cycle, but the amplitude of the oscillation may be significantly altered in cells that have sustained damage. For example, gamma-irradiation of mouse haematopoietic progenitor cells from bone marrow causes significant increases in the oscillation amplitudes of mRNA and protein of the CDK inhibitor p21 and the major negative regulator of p53, Mdm2. The time points in the circadian cycle when the damage had occurred may also significantly matter. If we may use the above example, gamma-irradiation of haematopoietic progenitors from mouse bone marrow occurring early in the night resulted in higher p21 and Mdm2 mRNA levels than the maximum levels of p21 and Mdm2 achieved when the irradiation occurred during the day [167]. The authors of the cited study did not find significant differences in the mRNA and protein levels of the pro-apoptotic proteins Bax or Puma in bone marrow cells irradiated during different phases of the 24-h cycle.

Differences in the amplitude of the circadian oscillations of the levels of expression of genes associated with response to DNA damage may also exist between cells of the same basic type at different phases of differentiation. Using the above example again, when the levels of the proteins p21, Mdm2, Bax and Puma were quantitated in cells isolated from peripheral blood instead of bone marrow cells, the magnitude of the increase in the levels of p21, Mdm2, Bax and Puma was almost two times higher after irradiation occurring during the day than the levels achieved after nighttime irradiation [167]. Blood cells that have been exported to peripheral blood are, in most cases, partially or terminally differentiated, having had their major proliferation-associated pathways already blocked at earlier stages of differentiation. Induction of apoptosis in damaged cells from peripheral blood is usually not associated with major short-term or long-term consequences for the organism, as they are normally rapidly replaced by new cells. Even in healthy individuals, there are haematopoietic progenitors circulating in the peripheral blood. Indeed, these cells are only a minor fraction, but they are capable of rapid proliferation. Therefore, it may be prudent that the cells with high proliferative potential that have sustained genotoxic damage at the times when the S-phase occurs (during the day) are routed to apoptosis en masse. On the one hand, their contribution as a source of new blood cells for the organism is negligibly small. On the other hand, if they succumb to neoplastic transformation, they may present potential danger. The risk for deleterious effects for the organism is likely to be higher if the blood cells that have sustained damage at times when replication is carried out are spared rather than disposed of. Haematopoietic progenitors in the bone marrow are,

however, precious. Their number is limited and their potential for production of new specialised cells unavoidably declines with accumulation of mutations (normal ageing, history of genotoxic exposures, etc.). It is likely that bone marrow haematopoietic cells that have sustained damage are preserved (rather than disposed of) by induction of cell cycle arrest and attempted repair of DNA damage, even at the expense of risk of introduction of mutations, which may later result in neoplastic growth, as these cells constitute the major source of haematopoietic precursors of the organism. Thus, nighttime irradiation of bone marrow cells induces cell cycle arrest so that repairs could be carried out, whereas daytime irradiation is likely to mount only the minimal requisite response (but causes a phase advance to the phase when checks of DNA integrity and repair normally occur).

6. Genotype-phenotype correlations for mutations and polymorphisms in genes coding for products of the core circadian machinery

The majority of the studies on the functioning of circadian clocks in vivo were carried out in animal models (Drosophila, rats and mice). Human studies on circadian rhythmicity are exclusively observational. Experiments involving specific setups that may cause disruption of circadian rhythms in human beings are considered unethical, as they entail prolonged periods of complete darkness and/or constant illumination, and because studies have so far shown that the health consequences may be quite serious. The reliability of currently available data about the impact of the disruption of circadian rhythm in humans may, therefore, be questionable. For example, in many earlier studies on the impact of light at night and insufficient lighting during the day the participants were allowed to switch artificial light on and off whenever they wanted to, despite its potential role in the resetting of the circadian clock [168, 169]. Findings from studies carried in rats and mice may be more reliable, but may not be directly translatable to humans. For one thing, the two species have radically different timing of the activity/rest cycles, and for another, the cell cycle and DNA repair in rodents may exhibit specific properties that are unique among mammals [117, 118, 170].

6.1. Animal models

Mouse models of deficiencies of virtually all genes of the core circadian loop (or variant alleles of these genes) have been established. Not all of these models exhibit altered circadian pattern (cycle shorter or longer than 24 h) when housed in the absence of environmental cues (e.g. constant darkness) or loss of circadian rhythmicity [reviewed in 171]. However, the phenotypes of the majority of the mouse models with defects in core clock genes include features of accelerated ageing (fur greying, atrophy of subcutaneous fat and muscle tissue, ectopic calcification, etc.) of varying severity and/or increased cancer-proneness. This was proposed to result from clock interference', that is, the deregulation of the circadian clock adjusts the unidirectional clock of ageing so that it runs' faster than its normal rate (at least for some tissues) [172].

The phenotype of *Bmal1*-deficient homozygotes is the most severe of all mouse phenotypes conferred by mutations in circadian clock genes. While loss of both copies of *Clock* or both copies of any of the *Per* and *Cry* genes does not disrupt the circadian rhythms in mice

housed constantly in the dark (although it may cause changes in cycle duration), loss of the *Bmal1* gene results in almost immediate and complete loss of circadian rhythmicity in constant darkness [173]. *Bmal1*-deficient homozygote mice are infertile and have severely shortened lifespan (3-6 fold decrease compared to the average lifespan of wild type mice). Bmal1 mouse mutants have lower body weight and lower organ weight, compared to wild type mice, and exhibit a phenotype consistent with accelerated ageing, characterised by early development of cataracts, arthropathy, ectopic calcification and atrophy of muscle and subcutaneous fat tissue [174–176]. Similarly to *Clock* mutants (see below), *Bmal1* mutant mice exhibit impaired glucose tolerance that gets progressively worse as age advances [177]. Mouse cells in which the expression of *Bmal1* is suppressed are incapable of inducing G1 cell cycle arrest upon p53 activation, as the activation of p53-inducible CDK inhibitor p21 is severely inhibited [150, 178]. Heterozygous *Bmal1*-deficient mice are prone to developing various tumours, spontaneously as well as induced by genotoxic agents (e.g. ionising radiation) [176]. The lifespan of the homozygous mutants is usually too short to assess the degree of their cancer-proneness adequately, but a predisposition to development of lymphoma after gamma irradiation was reported by the same authors. The production of glucocorticoid hormones and the response to stress show marked abnormalities in *Bmal1*-deficient mice [179].

Npas2 deficiency in mice results in slightly shortened circadian cycle without obvious features of accelerated ageing and/or cancer proneness [46]. Mice with mutant *Npas2*, however, may be incapable of synchronising feeding behaviour to food availability and may fail to entrain the circadian clock in response to food cues [180, 181].

Mice carrying mutations in the *Clock* gene experience lengthening of the circadian cycle and sleep phase delay [182, 183]. Homozygous carriers of *Clock* mutations have shorter-than-normal lifespan, decreased reproductive fitness and may exhibit traits consistent with accelerated ageing, such as early developing cataract and ectopic dermatitis [184–186]. Mouse *Clock* mutants have major issues with energy balance (food intake and energy expenditure). When fed ad libitum, *Clock* mutant mice would overeat, which is associated with development of a condition resembling human metabolic syndrome/diabetes type 2 that is characterised by early-onset obesity, hyperglycaemia and hyperlipidaemia (although the hyperglycaemia in Clock-deficient mice is not due to insulin resistance, as in metabolic syndrome, but, rather, to hypoinsulinemia, similar to the advanced stages of diabetes type 2) [187]. Clock-deficient mutant mice may also exhibit behavioural abnormalities, such as hyperactivity; as well as increased sensitisation to the effects of alcohol and cocaine [188].

Both genes coding for Per1 and Per2, and Cry1 and Cry2 must be disrupted at the same time to produce a phenotype of deregulation of circadian rhythms in the mouse, as the functions of the protein products in each pair of homologues may partially overlap [189, 190]. *Per1* homozygous deficient and *Per2* mutant mice do not exhibit significant alterations of cycle length [171]. Homozygous mice carrying mutations in the *Per2* locus, however, lose the orientation about the timing of food availability - that is, they are unable to predict and anticipate the time when food is normally available [191]. *Per2* mutant mice may develop accelerated ageing phenotypes after non-lethal doses of ionising radiation [148, 154, 192]. *Per2* mouse mutants have reduced lifespan and female *Per1*- or *Per2*-deficient or mutant

mice may have reduced reproductive capacity, whereas the males are generally not affected [171, 193]. Mice in which the copies of *Per1* and *Per2* genes have been knocked out may have differential responses to cocaine administered chronically depending on whether the one or the other gene has been knocked out. *Per1* knockout mice show little to no sensitisation to cocaine, whereas *Per2* knockout mice exhibit increased sensitivity [194]. Mice with targeted disruption of the *Per3* gene exhibit only subtle deregulation of the circadian rhythm [191].

Homozygous *Cry1* deficient mutants usually experience cycle shortening when housed in complete darkness, whereas *Cry2* homozygous mutants exhibit lengthening of the circadian cycle [reviewed in 171]. Mice with combined *Cry1*/*Cry2* homozygous deficiency immediately become arrhythmic in complete darkness [189]. A mild premature ageing phenotype manifested by lower body weight and increased incidence of dermatitis is typical of mice with double *Cry1*/*Cry2* deficiency. Liver regeneration after partial hepatectomy is impaired in Cry-deficient double mutant mice, with the expression peaks of cyclin B1, cdc2 (CDK1) and cyclin A2 occurring with 8–12 hours delay than the timing of the corresponding peaks in wild type mice [156]. The lifespan and the reproductive capacity of *Cry1*/*Cry2* double knockout mice are not significantly affected [reviewed in detail in 171].

Cry1 homozygous knockout mice, *Cry1*/*Cry2* double knockout mice and *Per2* homozygous mutant mice are cancer-prone (spontaneously or following genotoxic challenge) [176, 141]. The circadian pattern of expression of major genes coding for proteins involved in the control of the progression in the cell cycle (c-Myc, cyclin D1, cyclin A, Mdm-2) is grossly deregulated in mice with mutant *Per2* [154]. The increased levels of c-Myc are chiefly responsible for the cancer-prone phenotype of *Per2*-mutant mice and c-Myc overexpression is believed to be among the causes of the increased propensity for cancer observed in wild type mice with disrupted circadian rhythmicity [153, 195]. Rapid elevation of levels of c-Myc, however, may compel the damaged cell to take the apoptosis route directly, without preliminary damage assessment and induction of cell cycle arrest. The mechanism of tumour suppression by overexpression of *Per1* includes induction of the expression of c-Myc; downregulation of the expression of cyclin B and cdc2 (Cdk1), responsible for the G2/M checkpoint transition; and downregulation of the p53-inducible CDK inhibitor p21 [99]. The latter is associated with upregulation of some of cyclin-CDK complexes typical of apoptotic cells (cyclin A/Cdk2) [196, 197]. *Per1* and *Per2* genes are now officially classed as tumour suppressor genes [141, 198].

There is an intriguing interference between the deficiency of *Cry1*/*Cry2* in mice and the carriership of inactivating mutations in the *Tp53* gene. Both conditions, when inherited independently, result in cancer proneness and decreased lifespan [151]. Nevertheless, double mutant *Cry1*/*Cry2* mice that also carry inactivated *Tp53* gene copies may exhibit longer survival (cancer-free as well as overall) and somewhat extended lifespan than p53-deficient mice without *Cry* gene mutations. One of the current explanations of this phenomenon is that the deficiency of Cry proteins confers increased proneness to apoptosis by the p53-independent mechanism [151]. Thus, cells with damaged DNA are presumably eliminated early enough so that the risk for cancerous transformation is low.

6.2. Human phenotypes associated with carriership of polymorphic variants of core clock genes

Some rare disorders characterised by abnormal timing of sleep phase and/or duration may be associated with carriership of variant alleles of genes coding for proteins of the circadian clock. The Ser662Gly mutation in the human *PER2* gene (rs121908635, associated with advanced sleep phase syndrome 1) modifies a phosphorylation site in the PER2 protein [199]. Affected individuals exhibit very early sleep phase onset and offset.

The natural short sleeper' human phenotype is associated with heterozygous carriership of the mutation Pro385Arg in the *DEC2* gene [80, 200]. Carrier individuals require only about 5-6 hours of sleep per 24h, whereas the number of hours of sleep that the average healthy individual needs to feel rested are 7–9 per 24h. In carriers of *DEC2* Pro385Arg mutation, sleep phase occurs significantly earlier than what is considered an average time of sleep onset in the general population. As this mutation does not seem to be associated with any adverse effects, it is sometimes referred to as a normal polymorphism and not a mutation.

The C allele of the 3111C/T noncoding polymorphism (rs1801260) in the 3'-UTR of the human *CLOCK* gene has been associated with eveningness' (that is, feeling best and fittest in the hours of afternoon to evening/night, usually accompanied by sleep phase delay), as opposed to morningness', which is characterised by preference for the hours of early morning to early afternoon and sleep phase advancement [201, 202]. Other studies, however, had not confirmed that association [203, 204]. Carriership of the 3111C/T polymorphism in the human *CLOCK* gene was found to be associated with increased risk for adult attention-deficit and hyperactivity disorder (ADHD) [205]. Another single-nucleotide polymorphism in the *CLOCK* gene, rs10462028 (A/G) was found to be associated with increased risk for bipolar disorder in European populations [206]. The C allele of the rs2287161 (G>C) polymorphism in the human *CRY1* gene and the A allele of the rs10838524 (A>G) polymorphism in the *CRY2* gene are associated with increased risk for major depressive disorder in the Chinese population [207]. The association between carriership of the *CRY1* rs2287161 polymorphism and predisposition to major depression was found in European populations as well [206]. The same study also showed an association between the risk for major depressive disorder and the rs11123857 (A>G) polymorphism in the *NPAS2* gene.

An association between the variant (A) allele of the Rev-ErbA1 polymorphism rs2314339 (G>A) and the risk for abdominal obesity was identified in two different populations (specifically, of Spanish Mediterranean and of North American origin). Specifically, the risk for abdominal obesity was estimated to be about 1.5 times lower for individuals with A-allele containing genotypes (AA, AG) than in G-allele homozygous carriers. The Rev-ErbA1 rs2314339 genotype did not correlate with energy intake (eating patterns, dietary preference) [208].

Inherited polymorphisms in core clock genes may constitute a genetic factor associated with increased risk for development of various tumours. Carriership of the 3111C/T polymorphism in the human *CLOCK1* gene has been linked to increased susceptibility for colon cancer [209]. A variable repeat length polymorphism in the human *PER3* gene (4 or 5 copies of a 54-bp repeat polymorphism, rs57875989) [210] is associated with increased risk

for breast cancer in young women [211] The *NPAS2* Ala394Thr polymorphism (rs2305160) is associated with increased risk for B-cell lymphoma [212].

7. Association between the disruption of circadian rhythm and the risk of occurrence of common diseases and conditions (diseases of middle and advanced age)

Since many physiological processes occur rhythmically within the 24-h cycle, it could be expected that the disruption of the circadian rhythm may increase the risk for development of multiple diseases and conditions. Indeed, data from mouse models (see above) suggests that the dysfunction of the basic players in the constitution of the circadian rhythm may increase the risk for metabolic syndrome, diabetes, cancer and cardiovascular disease. The International Agency for Research on Cancer (IARC) published in 2007 a statement to the effect that "*... Shiftwork that involves circadian disruption is probably carcinogenic to humans...*" [IARC shiftwork statement]. Industrialisation is unavoidably associated with disruption of the day/night cycle as it increases the proportion of people involved in indoor jobs during the day (which means insufficient amounts of daylight) and staying up late at night in an environment saturated with artificial lighting. Typical evening activities in modern societies are usually associated with consumption of the major meal of the day late in the evening (or even the night) and increased frequency of snacking between meals (usually, on foods rich in carbohydrate and fat). To this adds the effect of night shift work and the opportunities for travelling long distances by air flight (associated with jet lag syndrome). It could be expected that the effect of disrupted circadian rhythms on human health would greatly increase in the near future.

Numerous studies conducted in humans showed that disruption of the physiological circadian rhythm may significantly increase the risk of various tumours - specifically, cancer of the mammary gland, the prostate gland, the colon and rectum; and the lung [213–218]. The relationship is at present best studied in hormone-dependent cancer, especially tumours of the female breast. Many of the studies of the impact of disruption of circadian rhythms on human health were conducted in females (typically, female health workers – specifically nurses, but also air flight attendants), and may, therefore, suffer from gender bias, but the fact remains that the risk for of breast cancer is 1.3–2 times higher in women that worked night shifts or took rotating shifts than in women that worked day shifts only, with the risk increasing with the increasing number of years spent in shiftwork.

The potential for adjustment of the core clock by external cues (light) may also play a role in the constitution of cancer risk. The overall incidence of cancer has been reported to be lower among the totally blind compared to the general population [219], with the risk for breast cancer in totally blind women being almost two times lower than the risk in the general female population [220, 221]. The risk for prostate cancer was also lower among the totally blind men than in the general male population [222].

The cancer risk conferred by disruption of circadian rhythms may be modifiable by other factors, including other risk factors. For example, there have been reports about increased

risk for lung cancer among selected groups with chronically disrupted circadian cycle (night shift workers) [217]. The risk, however, was modifiable by tobacco smoking.

Besides the impact on the risk for development of cancer, disruption of circadian rhythms may be partially responsible for differential outcomes when cancer has already developed. Studies conducted in mice demonstrated that light at night may stimulate the growth of carcinoma of the mammary gland [223, 224]. Results supporting the data from mouse models were obtained in human studies as well. Specifically, chronic bedtime misalignment was found to be associated with faster tumour progression in patients with breast cancer [35]. Tumour progression was also accelerated in mice subjected to experimental conditions imitating chronic jet lag [225]. Studies have shown that patients with metastatic colorectal cancer that exhibited normal or near-normal rest/activity cycles had significantly better performance status and higher survival rates than patients with disrupted day/night rhythm [226]. The patients wore wrist actigraphs at all times recording the activity at daytime and nighttime, and the dichotomy index (the ratio between activity when in bed and out of bed, I227]. Some authors have suggested that that the disruption of the circadian rest-activity cycle may be used as a simple prognostic marker for overall survival of patients with metastatic colorectal cancer and for performance status and quality of life [227, 228].

In some tumours (the already mentioned colorectal, breast and prostate cancers, but also in thyroid carcinoma) have been observed somatic mutations of the core genes of the circadian clock and/or the auxiliary clock genes [29–31]. Reduced expression of *PER1* and/or *PER2* genes was demonstrated in tumours of the pancreas, the kidney and head and neck cancers [229–231]. Decreased levels of PER1 and/or PER2 mRNA and protein in gastric cancer were found to be associated with poorer outcomes [232]. Experimentally induced downregulation of the expression of *Per2* in mice was shown to accelerate tumour growth *in vivo*, probably by upregulation of the expression of the positive regulators of the cell cycle cyclin D and E [233]; whereas overexpression of either *Per1* or *Per2* inhibited the growth of breast cancer cells and increased the levels of apoptosis among them [234].

The expression of *DEC1* is severely downregulated or the gene copies are deleted in over 50% of the cancers of the oesophagus [235]. No genetic mutations have been found in the tumours with decreased expression of *DEC1*; however, this may suggest epigenetic modifications [235]. Studies conducted in breast tumours showed that the promoters of *PER1*, *PER2*, *CRY1* and *BMAL1* genes were hypermethylated in >70% of the analysed tumours, whereas the rates of hypermethylation in healthy tissues were usually below 50% [29].

Studies conducted in patients with chronic lymphocytic leukemia (CLL) showed that in tumour cells the expression of *BMAL1*, *PER1* and *PER2* was downregulated, whereas the expression of *c-MYC* and cyclin D1 was upregulated [236–238].

The levels of expression of core clock proteins may serve as predictors of individual outcomes in cancer. High *BMAL1* levels in the primary tumour in patients with colorectal cancer were associated with longer overall survival (>1.5 times) than the overall survival rates in patients with tumours with low *BMAL1* levels [36]. Similarly, the progression-free survival in patients with colorectal tumours expressing *BMAL1* at high levels was estimated

to be almost twice as long as the progression-free survival in patients with tumours expressing *BMAL1* at low levels [36].

High levels of expression of *CRY1* in patients with colorectal cancer were associated with lower overall survival rates [239]. In CLL, the expression levels of CRY1 and the ratio between the expression levels of CRY1 and PER2 were associated with differential outcomes. Specifically, epigenetic silencing of the CRY1 gene was found to be associated with indolent disease [236, 237].

Disruption of the oscillating rhythm of expression of some of the core clock proteins may be associated with more rapid cancer progression. For example, homogeneous (non-oscillating) expression of *BMAL1* or *PER2* in breast cancers may be associated with increased rates of lymph node metastasis and more aggressive course of the disease [29].

It has been proposed that the growth rate of tumours was different at different times of the 24-h cycle [240]. Indeed, breast tumours grow in a time-of-day manner, with growth rates controlled by the circadian clock and deregulation of the expression of core clock genes may alter the growth rhythm of the tumour [233,241]. Per1 expression suppresses tumour growth at specific times of day (around the times when Per1 reached peak levels); while at other times of the day (when Per1 levels were lower) the tumours grow at more rapid rate [241].

The circadian clock has been found to play a role in the pathogenesis of multifactorial diseases and conditions other than cancer as well. As was already mentioned above, disruption of the circadian rhythm in animals and man has been linked with deregulation of feeding patterns/energy expenditure and with disordered glucose metabolism. Weight gain and/or failure to lose weight are common consequences of the disruption of the day/night rhythm [242, 243]. This occurs not only because of disordered food anticipation/food intake/energy expenditure but also because of decreased glucose tolerance and/or increased insulin resistance. In mice, plasma insulin levels were higher and the levels of glucose and triglycerides were lower in animals fed during the subjective day compared to mice fed during the subjective night [244]. Disruption of the circadian cycle in patients with diabetes type 2 is associated with increased insulin resistance and augmentation of inflammation markers [28]. Similarly, the decline in beta-cell function that is commonly seen in patients with advanced diabetes type 2 may be accelerated in patients with disordered day/night cycle [245, 246]. Diet and daily activity routines may interfere with the normal circadian rhythms. A diet rich in fats rapidly alters the rhythm of eating behaviour in mice and the correlation between the phases of the central clock and the peripheral clocks [247]. The same is valid, albeit the physiological effects are generally opposite, for physical activity (in mice – wheel-running) [248].

Environmental factors other than light, temperature and food intake may directly or indirectly affect the circadian rhythm. Such factor is, for example, tobacco smoke. *BMAL1* was rapidly acetylated and subsequently degraded in the lungs of mice exposed to cigarette smoke and in human patients with chronic obstructive pulmonary disease (COPD), compared with non-smoking mice and COPD-free human controls [249]. The disordered core clock rhythm may thus contribute to the inflammation and injury of the lung epithelium.

Normally, the pulse rate and blood pressure significantly decrease (dip) at night, when one's asleep. Individuals that do not experience the nighttime drop in blood pressure (non-dippers) are believed to be at elevated risk for vascular incidents [250, 251]. It is known from clinical practice that most of the cases of stroke, myocardial infarction and sudden cardiac death usually occur between 6 and 12am (after awakening and arising from night sleep) [252–254].

The short-term and long-term consequences of brain injury often include disruption of the day/night rhythm [255, 256]. The expression of *Bmal1* and *Cry1* is deregulated in mice with traumatic brain injury [257]. Stroke patients often exhibit disorders of the sleep-wake cycle after the incident [258, 259]. Neuronal susceptibility to ischaemia was found to be regulated by mechanisms directly dependent on the circadian clock [254]. *Per1*-deficient mouse models with cerebral ischaemia exhibited higher rates of neuronal injury than similarly injured wild type controls [260]. Cerebral ischemia induced at different times in the 24-h cycle resulted in differential levels of expression of initiator caspase-8 and -9 and executor caspase-3 [258]. Ischemic injury is associated with generation of very high levels of oxidative damage in DNA at the site of the lesion. Since DNA damage is capable of resetting the circadian clock, it is quite possible that the disruption of the physiological circadian rhythm following the damaging event may affect functioning of the system for assessment of the severity of DNA damage. Damaged cells deficient in core clock proteins, such as Per1 may be prone to judging the damage they have sustained as severe enough to trigger apoptosis, whereas wild type cells may assess the same amount of DNA damage as milder and possibly reparable [261].

The levels of cortisol and melatonin may significantly change before and after surgery, not only because of the activation of the stress response, but also because of specific effects of general anaesthesia. The sleep disorders commonly seen in postoperative patients are usually related to circadian rhythm disruption produced by deregulation of cortisol and body temperature homeostasis, as well as by direct deregulation of melatonin synthesis [262, 263].

Disruption of the normal circadian rhythm is a very common finding in mood disorders and the expression of core clock genes may be drastically altered in patients with depression [69]. The presence of sleep disturbances (insomnia at night with or without daytime sleepiness; or hypersomnia) occurring every or almost every night for least 6 months was listed among the DSM-IV diagnostic criteria for depressive disorders and was retained in DSM-V (www.dsm5.org). Abnormalities in the circadian fluctuations in body temperature and hormone secretion (glucocorticoid and mineralocorticoid hormones, sex hormones, etc.) are also common in patients in mood disorders [reviewed in 264]. The diurnal variation in cortisol levels may be specifically altered in depression. The dexamethasone suppression test is often abnormal in mood disorders (major depressive disorder and bipolar disorder, current phase depressed) [265, 266]. Higher evening levels of cortisol in patients with depressive disorders have been found to correlate with depression severity and the occurrence of psychotic features [267].

The circadian clock may be responsible for the diurnal oscillations in the inflammatory responses to acute infection. It is known that some of the markers for infection oscillate

during the 24-h cycle (e.g. different types of fever may regularly peak at the same time every day). This is usually attributed to features of the germinative cycle of the infectious agent. Nevertheless, it was shown that mice infected with Salmonella typhimurium showed higher rates of bowel and spleen colonisation when infected early in the rest period (in the morning) compared with mice infected in the evening [268]. The same study showed that mice infected during the subjective day developed higher-intensity inflammatory responses than mice infected during the night.

The circadian pattern of expression of core clock genes shows signs of gross deregulation in aged cells [269, 270]. Some of the approaches that are generally used to delay the onset of replicative ageing in cultured cells, such as reactivation of telomerase activity, may improve the rhythmicity and the amplitude of the circadian oscillation [270]. It has been hypothesised that the organic changes associated with ageing may cause relaxation of the control of the core circadian clock over the peripheral clocks (e.g. in the cardiovascular system, in the gut, etc.) [269]. As a result, different tissues of the aged organism may be in different phases of the circadian clock, resulting in deregulation of the expression of tissue-specific genes controlled directly or indirectly by the circadian clock. For the cardiovascular system such are, for example, plasminogen activator inhibitor-1 (a major regulator of endogenous fibrinolysis) and vascular endothelial growth factor (responsible for the neoangiogenesis in sites with atherosclerotic plaque and in tumour growth) [271, 272]. Disturbances in the rhythmical expression of these two genes may contribute to the risk for development of cardiovascular disease and cancer progression [269, 273–276].

The melatonin levels are decreased, the circadian rhythm in melatonin secretion is lost and rest-activity rhythm is grossly disturbed in patients with Alzheimer's disease [277, 278]. The rhythm of the expression of the core clock genes *BMAL1*, *CRY1* and *PER1* is lost in brains of patients with Alzheimer's disease from early preclinical stage onward [279]. According to same study, the levels of *CRY1* m RNA were elevated in patients with advanced Alzheimer's disease.

Excessive daytime sleepiness commonly seen in patients with Parkinson's disease is believed to be associated with disturbances in resetting of the master clock by light cues and functional disengagement of the secretion of melatonin by the pineal gland from the rhythmic signals set by the master clock, eventually resulting in decreased amplitude between the daytime and night time levels of melatonin [279–281].

8. Putting chronobiological concepts at work - chronological nutrition and cancer chronotherapy

8.1. Snack around the clock, or how chronological nutrition reinvented normal eating

With the emergence of the concept of healthy' or successful' ageing, there have been many suggestions about how to increase the chances of being among the lucky few' that preserve their mental and physical capacities until very old age. Some of these suggestions make sense, other are, at best, questionable [282]. There is a general agreement, however, that the essence of healthy ageing' is delaying the onset of the diseases of middle and advanced age until later (optimally – much later) than the average age of onset [283]. One of the

newer ideas for increasing the chances for successful ageing is chronological nutrition' – that is, staving off age-related diseases by following a nutritional regimen based on the rhythms set by the circadian clock [284–286]. As was already mentioned, there is significant association between the disruption of circadian rhythms and the development of glucose intolerance, cardiovascular disease and cancer. Therefore, the idea of chronological nutrition' makes a lot of sense, albeit it is nothing new. For example, it excludes eating at wrong times of the day' - e.g. late in the evening and at night, eating only one large meal per 24h and snacking between meals; as well as eating an unbalanced diet (e.g. rich in fats and/or carbohydrates). These are recommendations that almost any qualified nutritionist would give to healthy individuals as well as to people with specific diseases and conditions, regardless of their age and/or sex. The introduction and the popularisation of the concept of chronological nutrition', however, may increase the public awareness about the potential dangers associated with disruption of the normal rest/activity cycle and the risks of entrainment of the circadian clock by unwanted stimuli. Finally, it may supplement a lifestyle that might not prevent multifactorial disease, but may at least increase the chances for delaying their development and/or complications until later age.

8.2. Timing anticancer treatments to the circadian rhythm in order to achieve a good therapeutic response with minimum adverse effects - cancer chronotherapy

The risks for occurrence and the severity of the adverse effects in anticancer therapy may be different when the same genotoxic agents are administered at different times within the 24-h cycle. This is, in a nutshell, the basis of chronotherapy (abbreviated from chronomodulated therapy), one of the modern branches of anticancer therapy. As was already mentioned above, the timing of the different phases in the cell cycle in cancer and non-cancer cells of the same tissue may significantly vary (or even in cases when it varies only slightly, it might be just enough to make all the difference). Cancer chronotherapy attempts at synchronisation of the delivery of the genotoxic agent to the peak of DNA synthetic activity in cancer cells, so as to achieve maximum inhibitory effect on tumour growth and (hopefully) much less severe suppression of the proliferation of normal cells. Even in cases when the peaks of DNA synthetic activity tend to overlap between cancer and non-cancer cells of the same tissue, the (presumably) much higher proliferative activity of cancer cells would ensure that they are affected by the genotoxic agent more than normal cells. Chronotherapy is believed to be associated with lower risk for therapy-associated adverse reactions, or, if the adverse effects occur, they are expected to be milder [287–289]. As with chronological nutrition, the concept of chronotherapy is not a modern invention. The first documented cases of cancer chronotherapy in human patients intended to achieve maximum response with minimum adverse effects date back to the 70-ties of the XX century [290–292]. A detailed report about for one of these cases was published by William Hrushesky in 1998, although it occurred some twenty years earlier, in 1978-79. The patient was a 50-year old farmer woman treated repeatedly for metastatic epidermoid carcinoma. The patient was feeling quite well and went about her work as usual, despite the illness and the many treatments she received. Her major complaint was that every day she experienced severe skin itching that started around midday and became progressively worse as the afternoon progressed. Later, around 6pm, burning pain was added to the

itching that continued until the patient eventually fell asleep (after 10pm). She always woke up early in the morning (around 5–6am) free of any itching or pain and had no complaints until noon, when it started all over again. The patient consented to participate in a study that involved measuring the mitotic activity in tumour tissue and in samples taken from normal skin at different times of the day for several subsequent days. The mitotic index of keratinocytes was highest in the afternoon for both normal and transformed cells, but the mitotic index of cancer cells was several times higher than the mitotic index of normal skin cells. The treatment team concluded that a session of genotoxic treatments timed around 4pm is likely to affect all dividing skin cells, but the impact on the cancer cells would be significantly higher compared to the efficiency of the same treatment carried out in the morning, when the skin cells are usually still in G1 phase [107, 293].

As anticancer therapy developed steadily in the last decades of the XX century and the first decade of the XXI century, the hypothesis of scheduling genotoxic treatments to the times of the day when cancer cells were most susceptible to damage was supported with more experimental results. Nevertheless, definitive data illustrating the benefits from timed anticancer treatments began to be published only in the late 90-ties of the XX century. The largest body of information obtained so far is for metastatic colorectal cancer treated with oxaliplatin-5-fluorouracil-leucovorin combined regimens (FOLFOX) or irinotecan-5-fluorouracil- leucovorin (FOLFIRI). The first results of chronomodulated cancer therapy were highly encouraging. The objective response to therapy and the median survival were found to be significantly higher in patients treated with chronomodulated FOLFOX combined regimen for metastatic colorectal cancer than with conventional (flat-infusion) FOLFOX [294, 295]. The rate of serious therapy-associated adverse effects (grade 3/4) was found to be as low as 14-17 % in patients with colorectal cancer on the FOLFOX regimen when 5-fluorouracil and leucovorin were administered slowly from 10pm to 10am, peaking at 4am and oxaliplatin was delivered slowly from 10am to 10pm peaking at 4pm [296, 297]. Presumably, the properties of platinum as an adduct-forming agent were used during the day, the afternoon and the evening when the cancer cells were preparing for transition the G1/S checkpoint (where cells that have sustained too much damage are typically rerouted to apoptosis), and the 5-fluorouracil as a nucleotide-depleting agent was used at night, when the S-phase occurred.

Timing of delivery of irinotecan as a single agent was also found to be important for the individual tolerability of the drug (at present – in animal models). The tolerability of irinotecan in mice with osteosarcoma was found to be at its best at Zeitgeber time (ZT) 15, and at its worst at ZT3 [298].

Nevertheless, similarly to the relationship between the individual capacity for DNA repair, the response to anticancer therapies and the risk for toxicity effects; the association between the chronomodulated vs. conventional therapy and the outcomes in terms of response and adverse effects turned out to be not that straightforward as they initially looked. Some of the studies in patients with metastatic colorectal cancer receiving the same combined regimen (FOLFOX) did not elicit significant differences between the survival rates of patients receiving the same regimen in conventional and chronomodulated settings [299, 300]. The authors of the latter study hypothesised that the maximum tolerated dose that

was used affected the circadian clocks, thus decreasing the efficacy of the chronomodulated regimen.

The occurrence and the severity of adverse effects of genotoxic therapy may have radically different prognostic value as markers for therapeutic response in chronomodulated and conventional therapy. For example, it is known that the occurrence of adverse effects, such as neutropenia in patients receiving anticancer treatments is not uncommon and may be dangerous, even life-threatening. However, in patients on conventional chemotherapy neutropenia may be a predictor of good treatment response – presumably because high toxicity may indicate that the proliferation of tumour cells is also severely affected by the genotoxic treatment. It was reported that the development of severe adverse effects, such as neutropenia, fatigue and weight loss in the course of FOLFOX for metastatic colorectal cancer was associated with better survival in patients on conventional regimens, but poorer survival in patients on chronotherapy [300, 301]. Again, it was speculated that the circadian clock was possibly affected by the high doses of chemotherapeutic agents so that the right' timing of the delivery of the agent was offset from the time it was actually delivered.

There are also other unexpected effects of chronotherapy with regards to dissimilar responses in different groups of patients. Women often have better survival rates than men after various diseases and may have better responses to different therapies. This may be related to the fact that women usually stick to the prescribed treatment schedules and tend to drop off any kind of treatment less often than men. It is, therefore, notable that men were reported to fare better than women in the majority of studies of cancer chronotherapy, with respect to therapy-related toxic effects and the outcomes after therapy [297, 299, 300, 302]. This may be related to the fact that women experience other cyclically occurring changes as well, which may modify the rhythmicity of the circadian cycle. The basic parameters of cancer chronotherapies might need to be adapted for male and for female patients, or chronomodulated therapy ought to be preferred as a treatment option in male rather than female patients, at least until the mechanisms regulating the differential outcomes in men and women are made clear.

Targeted stimulation or suppression of the expression of proteins of the core circadian clock may find potential use in the therapy of common human diseases in the near future. It has been repeatedly reported that sensitivity to anticancer therapies may be modulated by the expression levels of core clock genes. In human cancer cell lines transfected with PER1 the proportion of cells in S-phase decreased rapidly, whereas the proportion of cells in G2/M phase increased [99]. Human colorectal cancer cells with downregulated expression of ATR exhibited increased sensitivity to doxorubicin when depleted of the TIM1 protein than the sensitivity achieved with ATR downregulation alone [145]. Induced overexpression of Bmal1 was shown to inhibit tumour growth and increase the sensitivity of human colorectal carcinoma cells to oxaliplatin [36]. This process is mediated by cell cycle arrest in G2/M phase presumably mediated by ATM, which has been considered as a cancer target [303,304]. Retinoid-related orphan receptors (RORs) have been shown to play roles in the regulation of lipid and steroid metabolism and the pathogenesis of cancer [305–307]. ROR-response elements were identified in the regulatory sequences of genes coding for some of the major apolipoproteins (APOA1, APOC2, APOA5 and others) [309–311]. Downregulation

of the expression of RORα is a common finding in tumour cells, specifically breast and lung cancer, which are known to be dependent on the circadian regulation [312, 313]. RORγ-deficient mice were shown to be short-lived and susceptible to lymphoma [314]. Thus, RORs have also been proposed as potential drug targets in the treatment of human disease in the near future [315, 316].

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